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1. Defective intracellular activity of GDP-D-mannose-4,6-dehydratase in leukocyte adhesion deficiency type II syndrome.

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Decreased availability of GDP-L-fucose in a patient with LAD II with normal GDP-D-mannose dehydratase and FX protein activities

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Abstract: Leukocyte adhesion deficiency type II (LAD II) is caused by a disorder in the metabolism of GDP-L-fucose, which causes hypofucosylation of glycoconjugates. This study analyzes a newly identified LAD II patient who shows the same severe hypofucosylation of glycoconjugates as the other described patients. However, *in vitro* assays of cytosolic extracts from leukocytes and fibroblasts of the patient demonstrated a normal GDP-L-fucose biosynthesis from GDP-D-mannose. Analysis of the two enzymes involved in the pathway, GDP-D-mannose 4,6-dehydratase and FX protein, revealed normal numbers of transcripts without any detectable mutations within the coding regions of either gene. In contrast to previously published observations [Sturla et al. (1998) FEBS Lett. 429, 274–278], the major pathway of GDP-L-fucose synthesis can be normal in LAD II. *J. Leukoc. Biol.* 66: 95–98; 1999.

Key Words: GDP-D-mannose 4,6-dehydratase · Sialyl-Lewis X · Bombay · fucose · infection

INTRODUCTION

Leukocyte adhesion deficiency type II (LAD II) was first described in two Arab boys aged 2.5 and 4.5 years by Etzioni et al. in 1992 [1, 2]. Until recently, no other patients have been identified. The two known children are characterized by recurrent infections with marked leukocytosis even in the interval between the infections [3]. They also have severe developmental delay, microcephaly with frontal lobe atrophy of the cerebral cortex, hypotonia, and retarded growth. The clinical phenotype is characterized by a flat and coarse face, a wide and depressed nasal bridge with anteverted nostrils, a protruding tongue, long eyelashes, and short limbs with broad palms.

The specific defect in LAD II is unknown but it must involve a general aspect of fucose metabolism because fucosylation catalyzed by many different fucosyltransferases is impaired. For instance, LAD II patients lack Sialyl-Lewis X (Sle^x), which is a fucosylated glycan found at the surface of neutrophil granulocytes. Without fucose, the glycan does not interact with E- and P-selectins on the endothelial cells, and leukocyte

extravasation into the tissues is severely impaired, causing an immunodeficiency [3]. The core structure of the erythrocyte blood group H-antigen is a fucosylated glycan but it is missing in LAD II, resulting in a so-called Bombay phenotype. Other fucosylated glycoconjugates are also absent or severely reduced in this disorder. Because many different fucosyltransferases are required, it was assumed early on that LAD II cannot be caused by a specific fucosyltransferase deficiency [1].

Fucosyltransferases require GDP-L-fucose. Ninety percent of it is synthesized intracellularly from GDP-D-mannose, and only 10% arises from salvage of degraded glycoconjugates or direct import [4] (Fig. 1). The fucosylation defect in LAD II could be caused by a defect in the GDP-D-mannose to GDP-L-fucose conversion. Two enzymes catalyze the conversion: GDP-D-mannose 4,6-dehydratase (GMD) and the FX protein (Fig. 1). The chromosomal localization [5], cDNA sequence [5–7], and transcript size [5, 6] are known for the genes coding for both human enzymes. The first enzyme, GMD, has 372 amino acids and depends on NADP⁺ as a cofactor [5]. The product of the enzymatic reaction catalyzed by GMD, GDP-4-keto-6-deoxy-D-mannose, is converted by the second enzyme, FX protein, to GDP-L-fucose. FX protein has 321 amino acids and builds a homodimer that has epimerase as well as reductase activities. NADH, or preferably NADPH, is needed as a cofactor for the reductase reaction [5].

The new LAD II patient analyzed in this study has the typical physical stigmata and laboratory findings as described for the other patients. At 14 months of age, the boy cannot sit and his body weight is less than 5 kg. He has the Bombay phenotype blood group and Sle^x is missing from his neutrophil granulocytes. Hypofucosylation of glycoconjugates is seen in all cell types investigated. Further details have been described elsewhere [8].

Fibroblasts and leukocytes from this patient were used to

Abbreviations: LAD II, leukocyte adhesion deficiency II; Sle^x, Sialyl-Lewis X; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase-polymerase chain reaction; HPLC, high-performance liquid chromatography; CBS, cystathionine β -synthase.

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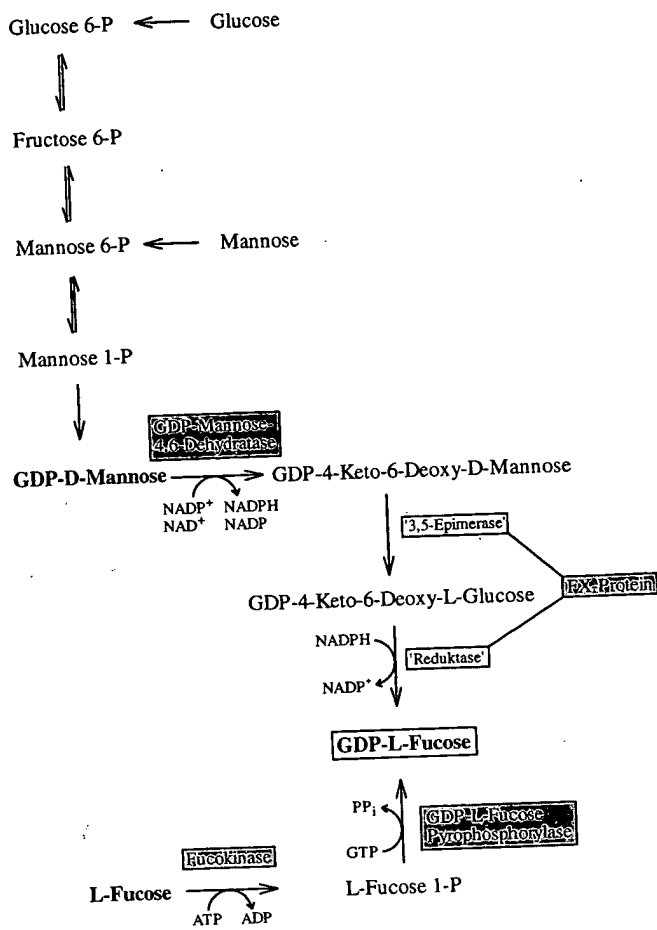


Fig. 1. Fucose metabolism. The two pathways of GDP-L-fucose formation.

investigate the major pathway of GDP-L-fucose biosynthesis. In contrast to a recent report [9], we provide evidence that GDP-L-fucose biosynthesis from GDP-D-mannose shows no abnormalities in LAD II cytosolic extracts. In addition, no genetic alterations were found for GMD and FX protein.

MATERIALS AND METHODS

Conversion of GDP-L-fucose to GDP-D-mannose

In vitro conversion experiments of GDP-L-fucose to GDP-D-mannose in cytosolic preparations from fibroblasts and leukocytes were done according to a method described earlier by different groups [5, 10, 11]. NAD^+ was substituted for NADP^+ in order to have comparable experimental conditions to a previously published biochemical study on LAD II [9]. Fibroblasts were grown as described previously [11]. Leukocytes were freshly prepared as follows: 5 mL blood anticoagulated with EDTA were laid over 5 mL water containing 2.1% (w/v) glucose, 1.5% (w/v) dextran, 0.33% (w/v) sodium citrate, 0.11% (w/v) citric acid, and 0.9% (w/v) sodium chloride. After sedimentation for 1 h at room temperature, the leukocytes were spun down from the supernatant. Hypotonic lysis of erythrocyte contaminations was done by incubating the cell suspension in 0.23% sodium chloride for 1 min. Fibroblasts were washed in phosphate-buffered saline (PBS) supplemented with 1 mM CaCl_2 and 1 mM MgCl_2 , taken up into 50 mM Tris, pH 8.0, containing 0.01% phenylmethylsulfonyl fluoride (PMSF) and homogenized by passing 15 times through a 22-gauge needle. The 100,000 g supernatant was taken as cytosol. When indicated, the cytosol was desalted by gel filtration over a PC 3.2/10 column on a SMART system (Pharmacia) with 50 mM Tris, pH 8.0, at a flow rate of 100 $\mu\text{L}/\text{min}$. *In vitro* conversion experiments were done at 37°C with 600 μg protein/mL in 50 mM

Tris, pH 8.0, containing 10 mM niacinamide, 5 mM ATP, 0.2 mM NAD^+ , 0.2 mM NADPH, 15 mM Mg^{2+} , and 7.5 μM GDP-[^3H]mannose (10,000 cpm). To trap the GDP-6-deoxy-keto sugar intermediates, NADPH and Mg^{2+} were left out from the reaction mixture. Reactions were stopped by heating the samples for 3 min at 95°C. Reaction products were characterized by high-performance liquid chromatography (HPLC) analysis on a C18 $\mu\text{Bondapak}$ column (Waters, Milford, MA; 3.9 \times 300 mm) with 0.5 M KH_2PO_4 as running buffer at a flow rate of 1 mL/min as described previously [13].

Reverse transcriptase-polymerase chain reaction (RT-PCR) and DNA sequencing strategy

Total RNA was prepared from both freshly isolated lymphocytes and cultured fibroblasts using the RNeasy system (Quiagen, Hilden, Germany). cDNA of oligo(dT)-primed total RNA was generated by AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany). For GMD and FX protein, the full-length coding regions were PCR-amplified: (1) GMD, GMD-1F (5'-CTCCGCCCTGCCGCCGAGCG-3', position -36) and GMD-1R (5'-GCAGCAGGGGCC GCAGGGGACC-3', position 1239), annealing temperature 64°C, hot start. (2) FX protein, FX-1F (5'-CTAGAACCCAGGTGCAACTGA C-3', position -22) and FX-R (5'-GGCACCTG ATCCTGTCTTCAG-3', position 991), annealing temperature 61°C, hot start. After gel purification, PCR products were cloned into pCR2.1 (Invitrogen Corp., DeShelp, The Netherlands). The inserts of purified plasmids were cycle sequenced using ThermoSequenase (Amersham, Braunschweig, Germany) with 5'-IRD-800 fluorescent-labeled M13 universal (5'-TGTAACACGACGGCCAGT) and reverse (5'-CAGGAAACAGCTATGACC) primers, respectively, and analyzed with the automated LI-COR 4000 sequencing device (LI-COR, Lincoln, NE).

Northern blot analysis

Total RNA prepared from cultured fibroblasts was separated on a formaldehyde-agarose gel and subsequently blotted onto a Hybond-plus membrane using capillary blotting as described by the manufacturer (Amersham). For the preparation of hybridization probes, specific PCR products were generated using the following primers and conditions: (1) GMD (132 bp), GMD-1aF (5'-GGCACCCGCCCTGCCCTCTG) and GMD-4R (5'-GCCGCCACGTTCTGGCCTTG), annealing temperature 65°C, hot start. (2) FX-protein (1014 bp), FX-1F, and FX-1R. (3) β -actin (308 bp), β -ACT1S (5'-CACCTTCTACAA(T/C)GAGCTGC) and β -ACT2AS [5'-TTCATGAGGTAGTC(G/A/T/C) GTGAG], annealing temperature 58°C. (4) Cystathionine β -synthase (1089 bp), HC-ex6 (5'-CCC TGG CTC ACT ACG ACA CC) and HC-C (5'-CGC CCA CGG CTC CGG ACT TC), annealing temperature 57°C. The probes were labeled by incorporation of Digoxigenin-11-dUTP during PCR (PCR DIG Probe Synthesis Mix, Boehringer Mannheim). Hybridization and detection was performed using the DIG Easy Hyb and the DIG luminescent detection system (Boehringer Mannheim).

RESULTS

In vitro conversion of [^{14}C]GDP-D-mannose into [^{14}C]GDP-L-fucose in cytosolic extracts

A high-speed supernatant was prepared from fibroblasts and incubated for various times at 37°C with [^{14}C]GDP-D-mannose, NAD^+ , and NADPH. Conversion of [^{14}C]GDP-D-mannose to [^{14}C]GDP-L-fucose was measured by HPLC (see Materials and Methods).

After 60 min, about one-third of the label was converted to GDP-L-fucose (Fig. 2). Control and LAD II extracts were equally active. When the cytosolic preparations were gel filtered to remove low-molecular-weight components, no products were formed on incubation. Addition of the cofactors for GMD and FX protein, NAD^+ and NADPH, to the desalted cytosol preparations initiated the conversion of GDP-D-mannose to GDP-L-fucose. If only NAD^+ was added as cofactor

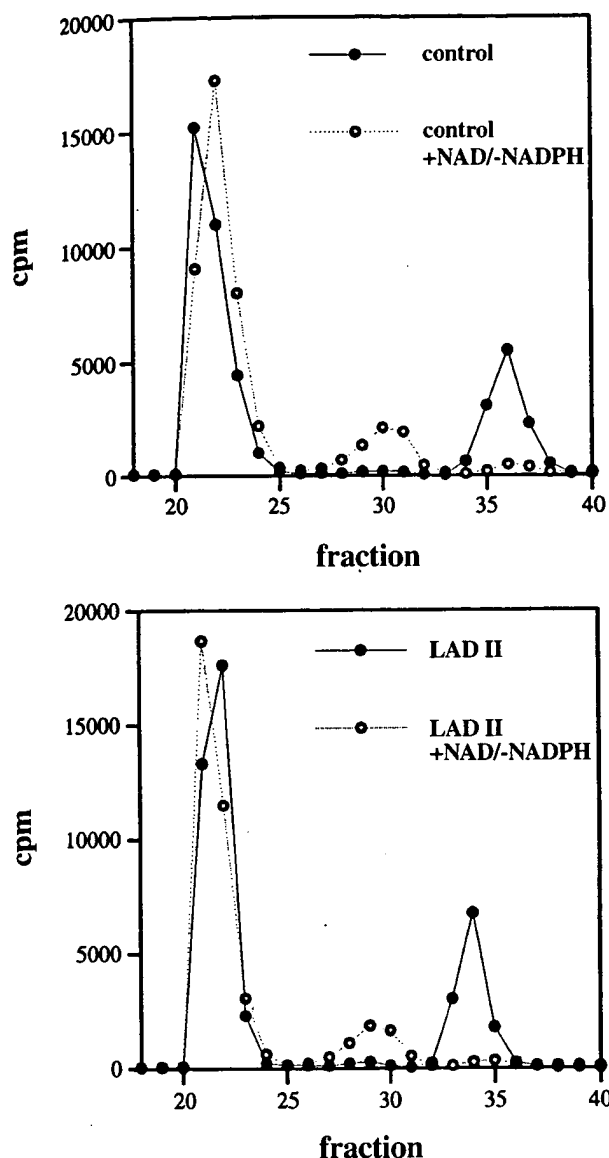


Fig. 2. *In vitro* conversion of GDP-D-mannose into GDP-L-fucose. [14 C]GDP-D-mannose (50000 cpm) was added to cytosol prepared from LAD II and control fibroblasts. NAD $^{+}$ and NADPH were added as cofactors to the reaction mix. Incubations were done at 37°C for 60 min. Metabolites were separated by HPLC as described (flow rate 1 mL/min, fraction size 250 μ l). After 60 min, [14 C]GDP-D-mannose (peak at fraction 22) was partially converted to [14 C]-GDP-L-fucose (peak at fraction 34–36). There was no difference between lysates prepared from the LAD II cells and controls (solid line). When small-molecular-weight components were removed by gel filtration and NAD and NADPH were subsequently added, the same, although less efficient, conversion was observed (not shown). When NADPH was left out from the reaction mix, an intermediate product accumulated (fraction 29–30) that corresponds to GDP-4-keto-6-deoxy-L-glucose (see Fig. 1). Again, no difference was observed between LAD II and control cells (dotted line).

for GMD activity, GDP-4-keto-6-deoxy-L-glucose accumulated as an intermediate (Fig. 1 and Fig. 2, open circles). No difference was observed between control and LAD II cells under any of these conditions.

The rate of *in vitro* conversion of [14 C]GDP-D-mannose to [14 C]GDP-L-fucose was linear and the same in control and LAD II cytosolic extracts (Fig. 3). There was no initial lag in controls or LAD II. This finding is in contrast to a recent report that

described a decreased activity and an abnormal kinetics for GMD activity in LAD II extracts [9].

Because leukocytes are known to have a severe hypofucosylation in LAD II, all experiments were also performed with cytosolic extracts from total leukocytes. Experimental results were identical to those described for fibroblast extracts (data not shown).

cDNA sequencing of GMD and FX protein

Total cDNA from fibroblasts and lymphocytes of the patient and his parents was prepared as described in Materials and Methods. RT-PCR generated cDNAs encompassing the whole coding sequence as well as the flanking 5' and 3' untranslated regions of GMD and FX protein were cloned into plasmid vectors. For GMD, the inserts of 10 independent plasmids were sequenced in total. A polymorphism at nucleotide position 798 (T > C) was detected in 50% of the clones generated from the patient's cells. Direct sequencing of the total cDNAs from the parents revealed homozygosity for the polymorphism in the paternal transcripts, whereas homozygosity for the wild-type sequence was present in the mother. Therefore, this intragenic polymorphism served to distinguish the two GMD alleles of the patient. No further genetic alterations were found either in the coding region or in the sequenced 5' untranslated region of each allele. Identical results were obtained when RNA was prepared from fibroblasts or lymphocytes, respectively.

Sequencing the cDNA of the FX protein, all clones from the parents and the child (total of 12) showed two sequence aberrations from the published sequence [7]. The two sequence aberrations were 315 G > C and 519 T > C. Neither of them causes an amino acid substitution. It is not known whether these nucleotide exchanges represent a polymorphism or represent errors in the published wild-type sequence. A polymorphism being present in only one of the two alleles of the patient was found at nucleotide position 306 (T > C) and again served to differentiate the two alleles. Full-length sequences of both alleles were obtained and revealed no mutation.

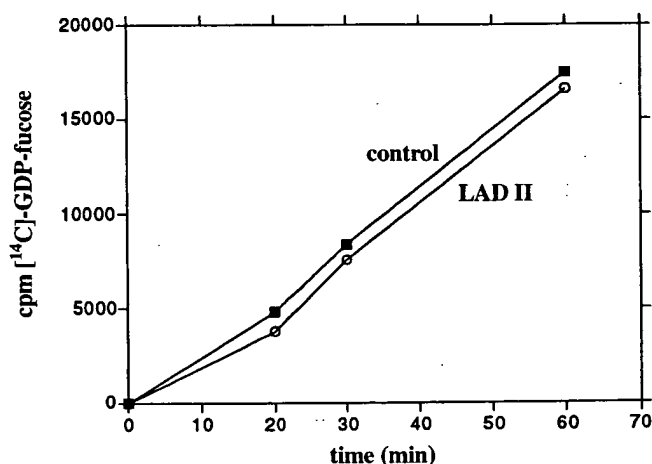


Fig. 3. *In vitro* conversion of [14 C]GDP-D-mannose to [14 C]GDP-L-fucose. Cell lysates were prepared from fibroblasts as described. [14 C]GDP-D-mannose was added and [14 C]GDP-L-fucose synthesis was assayed by HPLC after 0, 20, 30, and 60 min.

Northern blot analysis

The mRNAs of GMD and FX protein were analyzed by Northern blots. Probes for β -actin and cystathionine β -synthase (CBS) were used as reference markers. Normal amounts of transcripts of GMD and FX protein were found in fibroblasts of the patient (Fig. 4).

DISCUSSION

LAD II is caused by a severe hypofucosylation of glycoconjugates. Plenty of evidence suggests that GDP-L-fucose is limited in LAD II patients. Two possible explanations for this observation are that there is a defect in the biosynthesis of GDP-L-fucose or that GDP-L-fucose is not efficiently transported from its site of synthesis in the cytoplasm to the site of utilization in the Golgi.

A previous study analyzed the conversion of radioactive GDP-D-mannose to GDP-L-fucose in cytosol preparations from one LAD II patient [9]. Two abnormalities were found in the first reaction catalyzed by GMD. First, the reaction lagged for 60 min before reaching linearity. Second, the overall activity of GMD in LAD II was reduced even after prolonged incubation times. Our experiments showed no evidence for a defective conversion of GDP-D-mannose to GDP-L-fucose in LAD II, indicating a possible heterogeneity of this disorder.

Several mammalian cell lines have defects in the conversion of GDP-D-mannose to GDP-L-fucose [10, 11]. The CHO mutant Lec13 has a defect of GMD [11]. The dehydratase transcript is absent from these cells leading to an up-regulation of the FX protein mRNA level [6]. The mouse lymphoma cell line PL^R1.3 also has a GMD defect [10]. Cytosol preparations from either of the two cell lines show no *in vitro* conversion of GDP-D-mannose to GDP-L-fucose. This is in marked contrast to the unaltered *in vitro* conversion of GDP-L-fucose to GDP-D-mannose in our LAD II patient and must be due to a molecular defect different from the one present in this patient.

The experimental evidence suggests that LAD II is not caused by a defect in GDP-L-fucose biosynthesis. However, labeling of the LAD II fibroblasts and leukocytes with [³H]mannose revealed a profound decrease of fucose incorporation into newly synthesized glycoconjugates [C. Körner and T. Marquardt, unpublished observations]. It is interesting that the

mouse lymphoma cell line PHA^R1.8 PL^R7.2, shows a similar phenotype with reduced fucosylation from [³H]mannose and normal *in vitro* conversion of GDP-D-mannose to GDP-L-fucose [10].

Because it is well known that GDP-L-fucose causes a feedback inhibition of its synthesis from GDP-D-mannose [5], the decreased *in vivo* synthesis of GDP-L-fucose in LAD II and PHA^R1.8 PL^R7.2 could reflect an increased intracellular pool of GDP-L-fucose. A defect of GDP-L-fucose import into the Golgi could explain the phenotype of our LAD II cells and the PHA^R1.8 PL^R7.2 mouse lymphoma cell line. Whether this is indeed the case is the topic of current investigation.

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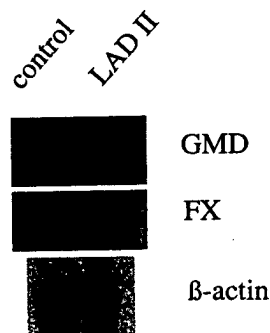


Fig. 4. Northern blot. Top panel, GMD; bottom panel, FX protein. Left lane, control; right lane, LAD II.